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Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells

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SUMMARY

Little is known of the role of T-cell mediated immune responses in the clearance and pathogenesis of respiratory syncytial virus (RSV) infection. In this study, we established persistent pulmonary RSV infections in athymic nu/nu BALB/c mice or immunodeficient irradiated BALB/c mice, and examined the patterns of virus clearance following adoptive transfer of splenic memory T cells. Primed T cells transferred between Day 5 and Day 8 of infection will clear lung RSV from both nu/nu mice and irradiated mice within 10 days of transfer. Partially purified Lyt 2^+ T cells are more effective than L3T4$^+$-selected T cells. No RSV-specific serum antibody could be detected, suggesting that clearance is by an antibody-independent mechanism. In contrast, delayed (Day 14) transfer of primed L3T4$^+$-selected cells clears lung RSV from nu/nu mice, and this correlates with RSV-specific serum antibody production. Clearance is not seen following Day 14 transfer of total primed T cells or T cells selected for the Lyt 2^+ subset.

INTRODUCTION

Respiratory syncytial virus (RSV) is a paramyxovirus responsible for severe respiratory tract infections in infants and young children, RSV pneumonia or bronchiolitis being a major viral cause of hospitalization within this age group. The need for an effective vaccine against RSV has been apparent for many years, but as yet none is available. Clinical trials of a formalin-inactivated RSV vaccine in the 1960s showed that the vaccine was effective in eliciting neutralizing and complement-fixing antibody responses, but that it failed to protect against infection and that the severity of subsequent disease was frequently enhanced (Fulginiti *et al.*, 1969; Kim *et al.*, 1969). A live RSV vaccine did not result in enhanced pulmonary disease upon natural infection, but was in other respects equally unsuccessful (Belshe, Van Voris & Mufson, 1982).

Both of these vaccine trials had indicated that neutralizing antibody may not be sufficient to protect against disease. The peak incidence of RSV infection is at 2 months of age (Parrott *et al.*, 1973), a time when significant levels of maternal antibody should still be present. Several groups have found that the presence of RSV-specific neutralizing maternal antibody does not protect the infant (Beem, 1967; Lamprecht, Krause & Mufson, 1976; Parrott *et al.*, 1973), although it may lessen the severity of the disease (Glezen *et al.*, 1981; Lamprecht *et al.*, 1976). Against these observations, there is nevertheless some evidence that protection is associated with the level of maternal antibody (Ogilvie *et al.*, 1981), and it has been noted that few infants under 1 month of age are infected (Parrott *et al.*, 1973), a period during which maternal antibody should be at peak levels. While the role of antibody in protection has been extensively studied, relatively little work has been carried out on cell-mediated immune responses in RSV infection. We wished to investigate whether T cells can play a role in the clearance of persistent RSV infections in an inbred animal model. Mice can be infected with RSV (Prince *et al.*, 1979), cytotoxic T cells (T_c) demonstrated in the lungs of the infected mice (Taylor, Stott & Hayle, 1985) and memory T_c found in the spleens (Bangham *et al.*, 1985). Persistent infections were established in athymic (nu/nu) or gamma-irradiated mice and the course of infection was monitored following adoptive transfer of various memory T-cell populations. We demonstrate here that the adoptive transfer of primed T cells can clear persistent infections in immunodeficient mice by two apparently distinct mechanisms: (i) clearance in the absence of RSV-specific antibody; and (ii) clearance correlating with the production of RSV-specific antibody.

Abbreviations: MHC, major histocompatibility complex; RSV, respiratory syncytial virus; T_c, cytotoxic T cell.

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MATERIALS AND METHODS

Virus

The human A2 strain of RSV was grown in HEp2 cells and stored in liquid nitrogen, as described elsewhere (Bangham *et al.*, 1985). This strain was used in all experiments.

Infection of mice

BALB/c mice were bred at the National Institute for Medical Research, London. For lymphocyte transfer experiments, donor mice 4–6 weeks of age were anaesthetized by intraperitoneal (i.p.) inoculation of 0·5 mg pentobarbitone sodium (May & Baker Ltd, Dagenham) and primed by intranasal (i.n.) inoculation of approximately 5×10^4 plaque-forming units (PFU) RSV. Recipient mice were 1–2-months-old BALB/c nu/nu mice (provided by the Institute for Research on Animal Diseases, Compton) or 6–8-week-old irradiated (450–550 rads, ^{60}Co source) BALB/c mice. The radiation dose was set according to the age and weight of the mice, the upper limit being the lethal dose for BALB/c mice (approx 600 rads). Recipient mice were also infected by the i.n. route.

Transferred T cells

Spleen cells were taken from normal mice or mice 1–4 months after priming by RSV infection. B cells were removed by direct rosetting of sheep red blood cells (SRBC) to which affinity-purified goat antibodies to mouse Ig had been coupled with chromium chloride. Briefly, 10^8 spleen cells (1 ml) were mixed with an equal volume of 5% coupled SRBC and rotated at 4° for 20 min. Rosette complexes settled out after standing for 5–10 min, followed by centrifugation at 400 *g* for 45 seconds. Residual red cells were removed from the supernatant lymphocyte preparation by lysis with 0·84% ammonium chloride solution. The yield was usually 25–30%, of which < 1% were B cells, as measured by direct immunofluorescence.

Lyt 2^+ T cells were depleted by treatment with an anti-Lyt 2.2 monoclonal antibody, HO2.2 (Gottlieb *et al.*, 1980), and guinea-pig complement (gpC). T cells (2×10^7/ml in Hanks' BSS) were incubated with 1/100 HO2.2 ascites fluid on ice for 60 min. After one wash the cells were incubated with 30% (v/v)gpC (in Hanks' BSS) at 37° for 45 min, and finally washed twice. Yields of T_h cells were usually 50–55%, with less than 2% contamination by Lyt 2^+ cells. T_h cells were depleted by an indirect rosette technique using a monoclonal antibody, YTS 191.1 (Cobbold *et al.*, 1984), against the L3T4 T_h cell-surface marker (Dialynas *et al.*, 1983). T cells (2×10^7/ml in Hanks' BSS) were incubated with 50 μg/ml YTS 191.1 (ammonium sulphate precipitated from ascites fluid) on ice for 60 min. These cells were then treated with coupled SRBC as described above, the L3T4$^+$ cells sedimenting in rosette complexes. Yields of Lyt 2^+ cells were 10–20%, with contamination by L3T4$^+$ cells being less than 2% of this preparation. This was the best level of depletion that could be achieved for both subset preparations. We found that further purification steps markedly reduced the yield of the desired subset without giving an improvement in purity. All lymphocyte populations were transferred by i.p. inoculation in 200 μl Hanks' BSS.

Virus isolation

Detection of RSV in the lungs of infected mice was carried out essentially as described by Taylor *et al.* (1984b). Lung tissue was removed from exsanguinated mice and homogenized in Hanks' BSS containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, antibiotics (60 μg/ml penicillin; 100 μg/ml streptomycin), 0·218 M sucrose, 4·8 mM glutamate, and 30 mM magnesium chloride to give 10% (w/v) suspensions. Homogenates were centrifuged at 9000 *g* for 1 min, and the supernatants stored in liquid N_2 before determination of infectivity by plaque assay. Titres are expressed as $\log_{10}$ PFU per pair of lungs (i.e. per mouse). A virus titre of $\log_{10} < 0{\cdot}7$ represents a negative result, meaning that no virus was detectable at the starting dilution. The plaque assay showed less than two-fold variation.

Antibody assay

Individual mice were tested for serum antibody to RSV by ELISA. RSV antigen was prepared by treatment of 10^7 (1 ml) RSV-infected HeLa or HEp-2 cells with 0·5% Nonidet P40 (BDH Ltd, Poole, Dorset) in phosphate-buffered saline (pH 7·0). Cell debris was removed by centrifugation (2000 *g* for 10 min) and the supernatant diluted 1/500 in distilled water. Uninfected cells provided a control preparation. ELISA microwell plates (Falcon, Cockeysville, MD) were coated with 50 μl dilute antigen and dried overnight at 37°. The plates were blocked with 5% pig serum and 0·05% Tween 20 in phosphate-buffered saline. Test serum samples (with positive and negative control sera) were titrated in 1/2 log steps and RSV-specific antibody detected with horseradish peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dako, High Wycombe, Bucks), using tetramethylbenzidine or *o*-phenylenediamine as the enzyme substrate. Antibody titres are expressed as $\log_{10}$ reciprocal of the end-point dilution. A titre of < 1·5 was regarded as negative.

Cytotoxicity assays

Memory T_c were detected in the spleens of recipient mice by stimulation of spleen cells *in vitro* with RSV for 5 days, followed by a ^{51}Cr-release assay, as described by Bangham *et al.* (1985). The SD of this assay was in the range 2–3%. Target cells were BCH4 cells, a BALC/c fibroblast line persistently infected with the Long strain of RSV (Fernie, Ford & Gerin, 1981), and uninfected BALB/c fibroblasts of the same line. RSV A2-specific T_c have been shown previously to lyse strongly BCH4 cells or targets infected with the Long strain of RSV (Bangham *et al.*, 1985).

RESULTS

Clearance of virus from persistently infected mice by RSV-primed memory T cells

Intranasal infection of normal BALB/c mice gives peak titres of lung RSV at Day 5, after which time the virus is rapidly cleared. To investigate the role of memory T cells in the clearance of RSV infection in mice, we have used a nu/nu mouse model system in which lung RSV has been shown to persist for at least 19 days (Taylor *et al.*, 1985). The persistence of RSV in nu/nu mice permits transfer experiments with RSV-primed memory T cells to be carried out, as shown in Table 1. Adoptive transfer of primed T cells 5 days post-infection resulted in the clearance of the lung virus by Day 15, but not by Day 10. Transfer of normal T cells failed to clear RSV from the lungs, although some reduction in virus titres was observed by Day 15 (Table 1). Assays for memory T_c in the spleens of RSV-infected mice showed that recipients of primed T cells possessed significant levels of memory T_c activity at Days 10 and 15, whereas T_c activity in the recipients of normal T cells was only seen at Day 15, with the same order of cytotoxicity as that seen 5 days after transfer of primed T cells (Table 2). Serum antibody assays by ELISA gave negative results at all time-points in these experiments.

Table 1. Lung RSV isolation following adoptive transfer of normal and primed T cells to persistently infected nu/nu mice

Cells transferred Day 5 post-infection†	Lung RSV titre* Day 5	Day 10	Day 15
None	3·4	<0·7	3·2
	3·4	3·6	3·0
	3·2	3·9	<0·7
	3·8	3·3	3·5
10^7 normal T		3·1	2·1
		3·5	1·4
		3·8	3·6
		3·3	2·8
10^7 primed T		3·2	<0·7
		3·0	<0·7
		3·2	<0·7
		2·0	<0·7

* Log_{10} PFU/pair of lungs, assayed on Days 5, 10, and 15 after intranasal infection with RSV.

† Prepared from spleen cells of normal mice or primed mice 1–4 months after intranasal infection with RSV.

Table 2. RSV-specific T_c memory in nu/nu spleens following T-cell transfers

Cells transferred Day 5 post-infection	Percentage lysis* Day 10	Day 15
None	−2	0
10^7 normal T	4	25
10^7 primed T	28	39

* Spleen cells were removed from host nu/nu mice on Days 10 and 15 after infection and restimulated *in vitro* with RSV for 5 days prior to the assay. Cytotoxicity was measured against RSV-infected BCH4 cells in a 3 hr ^{51}Cr-release assay at an effector:target ratio 32:1. There was no significant lysis of uninfected BALB/c fibroblasts.

In order to exclude possible local antibody formation in the lungs of recipient mice, we irradiated host mice. Intranasal RSV inoculation of BALB/c mice 48 hr after irradiation resulted in a persistent infection of the lower respiratory tract (Table 3). Transfer of primed T cells 5 days post-infection resulted in clearance of lung RSV in 4/5 mice between Days 13 and 17 (Fig. 1), indicating that primed T cells can clear a lower respiratory tract infection by an antibody-independent mechanism. Serum antibody assays up to Day 17 gave negative results.

Table 3. Lung RSV isolation from persistently infected irradiated mice

Mouse no.	Lung virus titres* Day 6	Day 16
1	4·4	4·3
2	5·0	4·5
3	4·9	4·5
4	5·1	+†

* Log_{10} PFU pair of lungs, assayed on Days 6 and 16 after intranasal infection with RSV. ELISA antibody titres were log_{10} 1·5 at both time points.

† Dead.

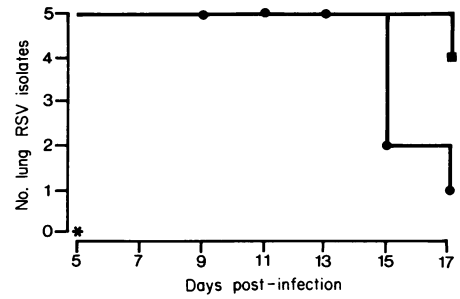

Figure 1. Time course of clearance of lung RSV from irradiated BALB/c mice. (■), RSV-infected control group (five mice). Mean titre of infected mice at Day 17; 3·9 log_{10} FFU/pair of lungs. (●), 10^7 RSV-primed memory T cells transferred on Day 5 (*) of RSV infection. Groups of five mice were assayed for lung RSV at 2-day intervals between Days 9 and 17 inclusive.

Clearance of lung virus by primed T cells depleted of L3T4$^+$ or Lyt 2$^+$ cells

To get an indication of which T-cell subset was effective in virus clearance, we purified subpopulations of memory T cells by treatment with anti-Lyt 2 antibodies and complement or by indirect rosetting with anti-L3T4 antibodies. These techniques strongly selected for the relevant T-cell subsets, but did not totally remove contaminating T cells. Selected L3T4$^+$ memory T cells from primed donors were less efficient at clearing the virus in infected nu/nu hosts than the total primed T-cell population (Table 4, exp. B). Three out of three mice still had lung virus, although the titres were lower than in control-infected host mice. RSV-specific serum antibody assays were negative in this experiment. We must point out that host spleen cells removed following transfer of the selected L3T4$^+$ cells generated some T_c after stimulation *in vitro* with RSV, though at a lower level than that achieved by the recipients of total primed T cells (data not shown). In contrast, a lower number of Lyt 2$^+$-selected T cells cleared lung virus in 4/4 infected nu/nu hosts

Table 4. Virus clearance following transfer of primed T-cell subsets to persistently infected nu/nu mice

Cells transferred Day 8 post-infection*	Lung RSV† Day 18	Ab titre‡ Day 18
Experiment A		
None	3·5 3·6 3·1 3·5	ND
9×10^6 total T cells	<0·7 <0·7 <0·7 3·7	ND
3×10^6 Lyt 2^+ selected	<0·7 <0·7 <0·7 <0·7	ND
Cells transferred Day 7 post-infection*	Day 17	Day17
Experiment B		
None	4·2 4·0 4·4 4·2	<1·5
8×10^6 total T cells	<0·7 <0·7 <0·7 2·8	<1·5
8×10^6 L3T4$^+$ selected	3·2 1·9 0·9	<1·5

* Prepared from spleen cells of primed mice 1–4 months after intranasal infection with RSV. L3T4$^+$ T cells were selected by treatment with anti-Lyt 2 antibodies + gpC, and Lyt 2^+ T cells were selected by indirect rosetting with anti-L3T4 antibodies.

† Log_{10}PFU/pair of lungs, assayed on Day 18 (exp. A) or Day 17 (exp. B) after intranasal infection with RSV.

‡ Log_{10} reciprocal end-point ELISA dilution. All mice gave negative results.

ND, Not done.

(Table 4, exp. A). The number of these cells transferred reflects their number in the primed total T cells transferred. The T cells selected for the Lyt 2^+ subset thus appear to be more efficient at virus clearance than those selected for L3T4$^+$ cells.

Delayed transfer of primed T cells to infected nu/nu mice alters the pattern of virus clearance

We wished to see whether transfer of primed T cells at a later time-point post-infection (14 days) would still result in virus clearance. A typical experiment is illustrated in Table 5, and shows a different pattern of virus clearance 10 days post-transfer (24 days post-infection). At this time-point we see antibody-dependent virus clearance following transfer of L3T4$^+$-selected

Table 5. Virus clearance following delayed transfer of primed total T cells and T-cell subsets to persistently infected nu/nu mice

Cells transferred Day 14 post-infection*	Virus titre† Day 24	Antibody titre‡ Day 24
None	3·3 3·6 3·4 3·8 3·9 3·5	<1·5
10^7 total T	0·9 1·7 3·7 3·9 2·4 2·8	<1·5
$2{\cdot}5 \times 10^6$ Lyt 2^+ selected	2·4 3·1 1·6 3·2 2·7 3·0 3·6	<1·5
8×10^6 L3T4$^+$ selected	3·7 <0·7 <0·7 <0·7 3·5 <0·7 <0·7	<1·5 3·4 2·2 3·0 1·6 2·5 2·1

* Prepared from spleen cells from primed mice 1–4 months after intranasal infection with RSV. L3T4$^+$ cells were selected by treatment with anti-Lyt 2 antibodies + gpC, and Lyt 2^+ T cells were selected by indirect rosetting with anti-L3T4 antibodies.

† Log_{10}PFU/pair of lungs, assayed on Day 24 after intranasal infection with RSV.

‡ Log_{10} reciprocal end-point ELISA dilution. All mice gave negative results, except for the group which received L3T4 selected T cells: individual Ab titres are therefore given for these mice.

cells in 5/7 mice. The total primed T cells and Lyt 2^+-selected cells fail to clear lung virus, have little effect on virus titres, and induce no RSV-specific antibody. Alterations in the nu/nu host immune system during the extended period of infection are thought to be responsible for these changes (see the Discussion).

It is unlikely that the virus titrations on lung homogenates from mice with RSV-specific serum antibody give false results as a consequence of virus neutralization *in vitro*. Neutralization experiments on lung homogenates from exsanguinated seropositive mice showed that any residual antibody failed to reduce the titre of a standard virus preparation, or inhibit plaque formation (results not shown).

DISCUSSION

It has been shown that antibodies can be protective against RSV infection. For example, passive transfer of monoclonal antibodies specific for the envelope F and G glycoproteins can protect against RSV infection in mice (Taylor *et al.*, 1984a) and

cotton rats (Walsh, Schlesinger & Brandriss, 1984). Similarly, convalescent sera from humans (Prince *et al.*, 1985a) or cotton rats (Prince, Horswood & Chanock, 1985b) can protect cotton rats from RSV infection. Although T_c have been found to clear many other virus infections, such as influenza (Yap, Ada & McKenzie, 1978; Lin & Askonas, 1981) and lymphocytic choriomeningitis (Byrne & Oldstone, 1984), the role of T-cell mediated immunity in RSV infection remains to be defined. Fishaut, Tubergen & McIntosh (1979) have described several cases of prolonged severe RSV infection in children with congenital deficiencies in cell-mediated immunity. In mice, RSV-specific, MHC-restricted T_c have been found (Bangham *et al.*, 1985; Taylor *et al.*, 1985) and natural cytotoxicity (that is not MHC-restricted) occurs in cotton rats (Kumagai, Wong & Ogra, 1985; Sun *et al.*, 1983).

In this study, using a model of persistent RSV infection in athymic or irradiated mice, we find that transfer of primed T cells clears infectious RSV in the lungs of host mice within 10 days, provided the hosts had been infected for 8 days or less. This clearance occurs in the absence of detectable antibodies. Normal T cells do not have this effector function. Primed T cells selected for Lyt 2^+ cells were also able to clear the virus, even in low numbers reflecting the Lyt 2^+-cell content of primed splenic T cells. In contrast, the L3T4$^+$-selected T cells were markedly less effective. We find here that it is not completely possible to remove contaminating memory T cells of a given subpopulation. Virus antigen appears to be powerful enough to select even a low number of precursor cells, as shown by the generation of some T_c function following transfer of T cells selected for L3T4$^+$ cells. For both T-cell subsets, further manipulations did not achieve greater purity and resulted in unacceptable losses, possibly giving an unrepresentative fraction of the desired subset. Although the memory T-cell subsets were not entirely pure, our results strongly suggest that Lyt 2^+ cells are more effective than L3T4$^+$ cells in clearing a persistent RSV infection in the absence of antibodies.

Our results also point to the influence of the timing of T-cell transfers in infected nu/nu mice. Fourteen days after RSV infection of host mice, a quite different pattern of immune responses is seen in reconstituted mice following the same T-cell transfers. Under these conditions, primed T cells selected for L3T4$^+$ cells cleared the lung virus in 5/7 mice and this clearance correlated with the production of RSV-specific antibodies. This antibody production may be due to generation of primed B cells in the nu/nu mice by Day 14 of infection, such that transfer of primed L3T4$^+$ cells leads to a rapid secondary-type antibody response. Induction of B-cell memory has previously been reported in thymectomized mice (Roelants & Askonas, 1972; Virelizier *et al.*, 1974), with secondary type antibody responses being observed following thymocyte reconstitution. Surprisingly, the primed total T cells or Lyt 2^+-selected populations at this late time after infection are unable to clear virus, nor is antibody produced. This suggests that these T cells, in conjunction with the persistently infected nu/nu host, induce some suppressive events (possibly involving the Lyt 2^+ subset) after transfer at Day 14. With regard to the apparent suppression of L3T4$^+$ function and antibody synthesis, it is interesting to note that Virelizier and colleagues (1974) found that thymocyte reconstitution of primed thymectomized mice suppressed a cross-reactive antibody response to purified influenza haemagglutinin.

In conclusion, the results of the early (Days 5–8) and late (Day 14) transfers of primed T cells indicate that two distinct and independent immunological mechanisms are capable of clearing persistent pulmonary RSV infections in immunodeficient mice. Early transfer of primed T cells leads to virus clearance by an antibody-independent mechanism, in which Lyt 2^+ cells appear more efficient than L3T4$^+$ cells. Transfers late in infection show that virus can be cleared by RSV-specific antibody in circumstances that appear to preclude RSV-specific Lyt 2^+-effector cell function. It is not known whether the antibody possesses neutralizing activity, although this is considered probable.

One of the key problems in the pathology of RSV is the elucidation of the immunological mechanisms involved in the exacerbation of natural RSV infection following vaccination with formalin-inactivated RSV, a phenomenon recently reproduced in cotton rats (Prince *et al.*, 1986). Transfer of T-cell clones of defined function and specificity to immunodeficient mice carrying persistent RSV infections should establish whether certain T cells are implicated in pathology or protection.

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